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14. ABSTRACT

Metastases account for nearly all deaths associated with advanced breast cancer and diagnosis of metastases results in less than a 3% survival rate over 20 years. There are five molecularly distinct subtypes of breast cancer, and it is unknown if primary tumors and metastases are genetically identical or if they differ in the expression of genes that facilitate cancer spread and survival. The purpose of this project is to identify approaches to prevent the spread of cancer cells and to inhibit the growth of cancer cells growing as metastases by utilizing overlapping in vitro, in vivo, and in silico models. Herein we identify that gene expression profiles of cancer cells and endothelial cells are largely maintained, regardless of the environment they are in. We also identify that the different breast cancer subtypes are predisposed to metastasize to different vital organs.

15. SUBJECT TERMS

Breast cancer, metastasis, microarray, coculture, endothelial cells, cell lines

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Table of Contents

Page

INTRODUCTION

The vast majority of deaths due to breast cancer for nearly half a million people annually worldwide are due to distant metastases in the lung, liver and brain[1]. Numerous studies have focused on breast cancer metastases and how they might differ from primary breast tumors; however, controversy remains regarding (1) the predisposition of specific classes of breast tumors to spread to distant sites and (2) the degree of similarity between primary breast tumors and their associated metastases. This project aims to understand how the different breast cancer subtypes (Luminal A and B, HER2-enriched, Basal-like, and Claudin-low) are involved in metastasis to vital organs. In the first year of the project, we began establishing the different model systems that we can use to better understand the genes that mediate the metastatic process. In this report, I will provide updates on the progress made for the second year of this project.

BODY

The approved statement of work for months 13-24 of this project is composed of two major components; A) Model Development and B) Specific Experiments.

Project/Aim1: Determine how the interactions of breast cancer cells with lymphatic-and blood vascular endothelial cells alters the aggressiveness of each intrinsic cancer subtype.

A. Model Development

Statement #1 (SOW AIM 1C). Months 12-18. *Isolate endothelial cells (EC) from xenografted and genetically engineered mouse tumors and characterize their in vitro growth requirements.* The goal here is to generate EC lines that are tumor-associated to contrast with the commercially available cell lines that were characterized in year 1 of this project.
Statement #2 (SOW AIM 1D). Months 12-18. *Development of a novel lymphangiogenesis-cancer cell interaction model.* If the [commercially available] ECs and model systems are not sufficient to study cancer-EC interactions we will modify a lymphangiogenesis assay recently developed to incorporate cancer cell-EC interactions.
Progress: Completed/NA. In year one, we established that commercially available human ECs could be utilized and are ideal to study how the interaction of human breast cancer cells affects the gene expression profile of each cell type. Our attempts to isolate matched sets of blood vascular (BEC) and lymphatic vascular endothelial cells (LEC) from mouse tumors resulted in unpure cell populations that primarily consisted of fibroblasts and cancer cells. Therefore, we have performed extensive co-culture experiments with matched sets of human BEC and LEC with human cancer cells that we will present below.

B. Specific Experiments

Statement #1 (SOW Exp #2). Months 6-18. *Migration/invasion assays.* A. LECs and BECs or their conditioned media (CM) will be incorporated into standard boyden chamber migration and/or invasion assays. ECs and their CM will be used to attract cancer cells. Conversely, luminal and basal human breast cancer cell lines will be used to

attract cancer cells (MCF7, ZR75, and SUM102, SUM149) will be used to attract the ECs. B. Once these assays are optimized, then the effects of hormones/antiestrogens and growth factors/growth factor inhibitors will be tested to ascertain if these pathways regulate ovserved cell motility. C. Alternatively to using boyden chambers, I will see cancer cells or ECs on glass coverslips and then transfer them to dish containing the other cell type. After incubation cells will be stained with cancer or EC markers (or viewed for GFP fluorescence) to identy motile cells.

Progress: Complete. Boyden chamber migration assays were utilized to test how EC secreted factors differently affected migratory ability of breast cancer cells **(Figure 1)**. Basal-like/Claudin-low cells lines (SUM149, SUM159, MDA-231) were all found to show extensive migration in response to EC secreted factors. Luminal cell lines (MCF7, T47D) and HER2-enriched (SKBR3) showed minimal, if any migration. In an attempt to identify the EC secreted factors that regulate this preferential movement of the estrogen receptor negative cell lines, we purchased recombinant proteins incorporated them into the assay. However, regardless of the concentration of the proteins (IL6, IL8, POSTN, BMP4, tPA, uPA, TBHS1, VEGFC, MMP1) none of them stimulated migration of any of the cancer cells. We conclude that none of the blood vessel secreted proteins by themselves are sufficient to induce migration of cancer cells. We hypothesize that either multiple cytokines are required to act synergistically to induce cell movement or the concentration/preparation of the commercially available recombinant proteins are insufficient in this assay.

Statement #2 (SOW Exp #3). Months 12-24. *Morphological changes (tube formation assays).* Establish 2D and/or 3D tube formation assays with ECs. Incorporate luminal and basal cancer cells lines into these assays to ascertain the effects of cancer cells on EC tube formation (rate of formation, # of branches).

Progress: Complete. We tested how luminal and basal cells effect endothelial cell proliferation in two ways. First, after developing a tube formation assay, we tested how media that had been conditioned by either MCF7 (Luminal) or MDA231 cells would affect the number of closed loops formed. However, growth of BECs in media other than the standard EC growth media resulted in a substantial decrease, or complete lack of, loop formation **(Figure 2)**. Therefore, all cells were grown in the endothelial cell media for 24 hours and conditioned media from each cell line was applied to BECs. Other than an overall reduction in tubes formed with all conditioned medias, we observed no significant differences with Luminal or Basal-like cell line conditioned medias. We attribute this overall reduction to lack of appropriate growth factors and serum in the conditioned medias.

Statement #3 (SOW Exp#4). Months 6-24. *Proliferation*. 1. A-Optimize Promega's CellTiter96 non-radioactive cell proliferation assay. B-Determine the effect of CM from both EC lines on proliferative ability of luminal and basal breast cancer cells. C-Expand findings in B to determine how hormones, growth factors, and their inhibitors influence cancer cell proliferation. D-If feasible, determine effects of CM from cancer cell lines on EC proliferation. If the CellTiter assay results need expansion perform 2-72hr BrdU labeling co-culturing experiments with cancer cells and ECs.

Progress: Complete. We are using the same approach as used for the migration assays described above in that we are using MCF7 and SUM149 for our luminal and basal breast cancer cell lines and BEC/LEC. Interestingly, in contrast to the migration results we observed that EC

conditioned media stimulated vast proliferation of the Luminal/HER2-enriched cell lines and modest increases in proliferation with the Basal-like/Claudin cell lines **(Figure 3)**. In an attempt to identify the EC secreted factors that regulate proliferation we tested the recombinant proteins that were tested in the migration assays presented above. Similar negative results were found suggesting that multiple EC secreted factors are required to stimulate cancer cell division. However, two recombinant proteins, IL6 and IL8, did show modest effects on the Luminal and Claudin-low cell lines respectively. Thus these two proteins may be good targets to inhibit the growth of these two cancer types and future studies may aim to inhibit these pathways with i*n vitro* and *in vivo* experiments.

Statement #4 (SOW Exp#4&7). Months 6-24. *Transcription*. Perform mono- and coculture microarray experiments. Assess estrogen and growth factor regulation of genes in cancer cells under both conditions.

Progress: Complete. In year one, we assessed how direct culturing of cancer cells with ECs effects gene expression of cancer cells. The downside to this approach is that we were uncertain which cell type, the EC or the cancer cell was producing the transcript of interest. Therefore, we modified these assays to include a transwell assays. These devices are similar to the migration assays described above, except the pore size in the transwell membrane is too small to allow the cells to migrate through, yet secreted proteins can transverse the membranes and act on the other cell line. In these assays the cancer cells were plated on plastic and the ECs were placed in the transwell. This would allow us to only isolate cancer cell RNA for subsequent microarray analyses on the same cell lines utilized for migration assays discussed above. In addition, we also tested how the various cancer cells effected EC gene expression, and the genes that are modulated by secreted factors are shown in **(Figure 4)**. Note there is an overall maintenance of all cancer cells and ECs. One of the major goals was to find common pathways within the various Luminal or Basal-like/Claudin-low cell lines that were regulated by EC secreted factors. However, in the few genes that were significantly altered, we found very little overlap in the modified genes, suggesting that even though the cancer cell lines may be of a similar subtype, there is extensive heterogeneity in the response of different cancer cells to endothelial cells. Similar results were found with the EC responses to cancer cells. However, we did find that the interaction of both BECs and LECs with MCF7 cell conditioned media resulted in a significant upregulation of genes that are known to be involved in the interferon response pathways. This confirms data from a recent publication showing similar results[2]. Overall, there were only modest changes in the gene expression profiles of the cancer cells and the ECs in response to the other cell type. For example, there was no shift of the luminal cancer cells to become more Basal-like when exposed to EC secreted factors and vice-versa **(Figure 4)**.

In the proliferation results presented above, we found that IL6 regulated MCF7 (Luminal) proliferation whereas IL8 upregulated SUM159 (Claudin-low). Therefore we performed gene expression microarrays on these cell lines with or without treatment of the respective recombinant proteins. Surprisingly, even though both these proteins functioned to increase proliferation there was little overlap in the genes commonly upregulated. These studies will continue into the third year of funding, as we will attempt to identify the best target genes to overexpress or knockout to inhibit cancer cell proliferation.

Project/Aim2: Elucidate mechanisms through which metastatic microenvironments reduce breast cancer responses to therapeutics.

Statement #5 (SOW EXP #1B). Months 0-36. Use our microarray database of more than 350 samples to investigate differences in human breast tumors and their metastases.

Progress: In progress, on schedule w ith SOW. This SOW has received extensive attention over the past 12 months and we have made major progress in understanding the genomics of metastasis. In the first set of experiments we aimed to understand how different primary tumors and metastases are from each other. Fortunately, we have an extensive database of human breast tumors and metastases gene expression microarrays, some of which are patient-matched. Therefore, we performed intraclass correlation (ICC) analysis on different groups of arrays. To further examine the degree of relatedness of breast tumors and their metastases, we performed correlation analysis using thousands of genes, and hundreds of pre-defined gene expression signatures/models[3] using a large set of tumors and paired metastases. Intra-class correlation (ICC) values were determined between pairs of samples using multiple classification/grouping methods: 1) different pieces of the same primary tumor ("intrinsic pairs"), 2) tumors and their matched metastases (all metastases, or further separated into either lymph node (LN) or distant), 3) tumors and their matched metachronous metastases, 4) sets of synchronous metastases from the same patient, 5) tumors from different patients grouped by intrinsic subtype, and 6) metastases from different patients **(Figure 5A)**. On average there was a very high concordance between two pieces of the same primary tumor (ICC=0.9 [0.89-0.91]), while pairs of tumors and their metastases exhibit lower concordance values (0.82 [0.8-0.83]). As observed by the metachronously paired tumor-metastasis samples, gene expression did not change substantially over time. The autopsy patient data (0.72 [0.68-0.75]) suggests that normal organ RNA may be the variable most responsible for the decreased similarity between tumor-metastasis pairs. This hypothesis was supported by increased ICC values of 20 matched pairs of laser-captured tumors and LN metastases[4] (0.9 [0.85-0.94]). Individual gene measurements can be fraught with "noise". Thus, to further test the relationship between tumors and metastases, ICC values were identified using a compendium of 298 different gene expression signatures/modules[3], where each module is a summary measure of tens to hundreds of gene. The overall ICC values were higher than individual genes (thus showing greater robustness for modules) and the breast tumor-metastasis pairs showed high conservation of pathways **(Figure 5B)** . The signatures with the most variability between tumors and matched metastases were associated with extracellular matrix (ECM) proteins, which likely arise from different amounts of fibroblasts**.**

Since the majority of genes maintain their RNA expression levels when growing as either primary tumors in the breast or as metastases, we sought to determine if the different intrinsic subtypes showed a predilection for metastasis to specific organs using genomic data arising from primary tumors only. Therefore, we combined four public microarray datasets with Distance Weighted Discrimination[5], providing 855 tumors with documented first site of relapse[6-9]. Principal Components Analysis found that the overall variation of gene expression was due to the biology of the tumors, and not by cohort or microarray platform **(Figure 6)**. Status for ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was recorded for 852, 537 and 499 tumors, respectively, and of the 482 tumors with defined status for all 3 markers, 110 were triple negative (TN); Kaplan-Meier analyses for site of relapse with these markers are shown in **Figure 7**. For all sites of relapse, ER/PR negativity was associated with

increased metastases, except for bone, in which both ER+ and ER- tumors recurred. Clinical HER2+ and TN status were associated with liver and brain/lung relapse, respectively.

Next, each tumor's intrinsic subtype was calculated for this combined data set using the PAM50[10] and the Claudin-low predictors[11]. Of the 855 tumors, 76 were identified as Normal Breast-like, and since this tumor classification is reflective of mostly normal breast tissue[10] these tumors/samples were excluded from further analyses, leaving a dataset of 779 tumors. Based on the site of first relapse data for liver, lung, brain and bone, Kaplan-Meier plots were generated, and subtype was correlated with site of relapse **(Figure 8)**. Compared to Luminal A, Basal-like and HER2-enriched tumors showed the highest hazard ratio (HR) of relapse to any site (Basal HR =2 [1.5,2.8]; HER2-enriched=2 [1.4,2.7]) followed by Claudin-low (1.6 [1.1,2.3]) and Luminal B (1.6 [1.2,2.1]) tumors. Important findings included that: 1) bone metastasis was the most common—regardless of subtype **(Table 1)**, 2) brain relapse occurred most frequently in non-luminal samples, 3) liver relapse was associated with HER2-enriched tumors, and 4) lung relapse occurred often with Claudin-Low and Basal-like subtypes. In all analyses, Luminal B were more aggressive than Luminal A tumors, thus providing a useful stratification for ER+ tumors.

In 2009, Bos et al[7] utilized two human breast cancer cell lines, CN34 and variants of the MDA-MB-231 human breast cancer cell line (a Claudin-low cell line), along with gene expression data from human breast tumors, to identify 17 genes whose expression correlated with brain relapse (BrMS). Given the clear associations observed for the intrinsic subtypes and sites of metastases, we hypothesized that the BrMS would correlate with Basal-like and/or Claudin-low subtypes. ANOVA from two different datasets supported this hypothesis (**Figure 9)** . Massague and colleagues also developed a Lung Metastasis Signature (LMS)[12] , which was also associated with intrinsic subtype **(Figure 9)**.

Recently a genomic method to quantify breast epithelial cell differentiation status, known as the DS predictor[11], was developed. This predictor is based on the genomic signatures of FACS purified populations of mammary stem cells, luminal progenitors, and mature luminal cells of the normal human breast[13]. DS are based on the premise that mammary stem cells are the least differentiated cells in the breast and they give rise to Luminal progenitors, which then produce mature Luminal cells; for the DS, higher scores represent greater differentiation along the axis which culminates in mature ER+ luminal cells. In this spectrum, Claudin-Low tumors are the least differentiated, followed by Basal-like, HER2-enriched, and ending with Luminal A and B[11]. Since Claudin-low and Basal-like tumors were associated with brain relapse, we postulated that the more undifferentiated a tumor is on this axis, the more likely it would be to metastasize to the brain. To test this hypothesis, gene expression data from parental and organ-tropic (brain, lung, and bone) MDA-MB-231 cell lines were obtained from the Gene Expression Omnibus, and their DS calculated and plotted on the DS axis **(Figure 10A)**. Shown on the same scale are the 779 breast tumor dataset **(Figure 10B)**, cancer cell lines of various tissue origins (NCI60)[14] **(Figure 10C)**, and the MDA-MB-231 series[7,12,15] **(Figure 10D)**. Overall, Claudin-low and Luminal breast cancer cells lines show the same relative differences in differentiation status as is seen in primary tumors. Importantly, the MDA-MB-231 cells from the NCI60 and Massague studies showed nearly identical DS, and the brain-tropic MDA-MB-231 cells were significantly less differentiated than the parental cell line.

To identify other features shared between low DS tumors and brain metastasis, we analyzed the NCI60[14] cell line series. Interestingly, DS were found to be similar in Claudin-low breast cancer cell lines, central nervous system (CNS) cell lines, and another tumor type that aggressively spreads to the brain, melanoma[16] **(Figure 1 0C)**. To identify genes that mediate cerebral colonization Significance Analysis of Microarrays (SAM) was performed on these three cancer cell line types versus the rest of the NCI60 lines and 265 genes were identified as significantly highly expressed (FDR=0%). Ingenuity Systems Pathway Analysis found that 'cellular movement' was the top biological function associated with these genes.

We next sought to better understand the information that DS provides for predicting site of metastasis. Since there is a range of differentiation within each intrinsic subtype **(Figure 10B)**, we tested if the least differentiated Basal-like/Claudin-low tumors were more metastatic than the more differentiated Basal-like/Claudin-low tumors. Kaplan-Meier analysis and Log-rank tests determined that the least differentiated half of these tumor subtypes were associated with significantly more relapse to brain (p=0.002) and lung (p=0.024). This same approach applied within Luminal and HER2-enriched tumors found no association of DS with bone or liver relapse, thus this association appears specific for brain and lung.

To directly visualize the information that DS and intrinsic subtypes provide for predicting site of metastasis, we plotted the DS of the 779 tumors versus the Hazard Ratio (HR) for each site of metastasis **(Figure 11A)**. The tumors were then ordered based on DS and all genes (11,068) hierarchical clustered **(Figure11B)**. Interestingly, the lowest DS have a much higher HR for brain and lung metastases, and this risk drops off quickly as differentiation increases.

These in silico analyses will continue into the third year of funding, and we anticipate a high impact publication in 2011.

Statement #6 (SOW EXP #1B). Months 0-24. Develop tumor cell lines from GEM (genetically engineered mice) tumors, label with GFP-Luc. Using these GEM cell lines and the human GFP-Luc lines established above, grow mammary gland tumors, isolate tumors and metastases for microarray and immunohistochemical analyses.

Progress: Complete. Additional experiments are planned for year 3. We have done extensive experimentation with breast cancer cell lines that represent the different intrinsic subtypes. One of the cell lines has provided extensive metastasis *in vivo* that we have characterized with gene expression microarrays. **Figure 12** shows a hierarchical cluster of gene expression microarrays from ZsGreen-tagged T11 mouse cancer cells grown as cell lines, xenografted mammary gland tumors, or as metastases. Interestingly, extensive differences in gene expression were observed in the primary tumors and the metastases. However, these differences appear to be primary due to normal host organ cells contributing to the results of the microarray. For example, the 5th sample from the right is a normal liver sample, and the three samples to the right of it are liver metastases. By contrasting the liver metastases with the normal liver and the pure T11 cell line, it is apparent that the mouse liver is contributing significantly to the microarray. Therefore, in year three of these studies we plan to either laser-capture out the cancer cells prior to array analyses, or use a subtraction based approach to remove genes that are contributed by the normal host organ.

KEY RESEARCH ACCOMPLISHMENTS

1. Identified that overall gene expression profiles of cancer cells are maintained when cancer cell lines interact with blood vascular and lymphatic vascular endothelial cells.
2. Identified that the breast cancer intrinsic subtypes metastasize to specific vital organs.
3. Identified that tumor Differentiation Score can be used to identify tumors likely to spread to the brain and lung.

REPORTABLE OUTCOMES

1. Applied for a University of North Carolina Cancer Research Fund Grant (12/2010).
2. Developed extensive databases of human gene expression microarrays; cancer cell line-endothelial cell cocultures, human tumors and metastases.
3. Prepared a manuscript for submission to Nature Medicine in early 2011.

CONCLUSION

Year two of the funded research project has provided an extensive understanding of the genomics of metastasis. We found that overall, cancer gene expression profiles are maintained in cancer cell lines, regardless of if they are by themselves or associating with endothelial cells. This builds on a previous publication that found that cancer gene expression profiles only modestly change when cancer cells are grown in two versus three-dimensions[17]. Further evidence supporting this maintenance of overall gene expression, regardless of tumor microenvironment, was found from our *in silico* analyses of human tumors and metastases. We found that nearly all genes, and broader gene expression signatures, are maintained in tumors and metastases. Both of these lines of research support our hypothesis that knowing a tumor's intrinsic subtype is critical for proper treatment and prevention of metastasis.

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APPENDICES

Name: **Joshua 'Chuck' Christopher Harrell** Current Title: Postdoctoral Fellow

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
University of Colorado Health Sciences Center-Aurora, CO, USA	PhD	2007	Cell & Developmental Biology; Cancer Research
North Carolina State University-Raleigh, NC, USA	BS	2000	Biological Sciences

RESEARCH AND PROFESSIONAL EXPERIENCE

University of North Carolina at Chapel Hill, NC, USA
Guest Lecturer/Teacher Assictant, Biology 101 Lab (8/2010-current)
Postdoctoral Fellow; (8/2008-current)
Lineberger Comprehensive Cancer Center Postdoctoral Advisory Committee (8/2008-current)
University of Colorado Health Sciences Center, Aurora, CO, USA
Postdoctoral Fellow (5/2007-6/2008)
Graduate Student (8/2002-5/2007), Doctoral Candidate (11/2005-5/2007)
University of Adelaide, Adelaide, South Australia
United States National Science Foundation EAPSI Participant (6/2007-8/2007)
National Institutes of Environmental Health Sciences, Research Triangle Park, NC, USA
Intramural Recreational Training Award (12/2000-7/2002)
Undergraduate Internship (9/1998-6/1999, 8/1999-12/2000)
North Carolina Division of Marine Fisheries, Morehead City, NC, USA
NC State Government Undergraduate Summer Internship Program (6/1999-8/1999)

AWARDS

United States DOD Breast Cancer Era of Hope Postdoctoral Training Award (1/2009-12/2011); $375,000
Lineberger Comprehensive Cancer Center Postdoctoral Fellowship (10/2008-10/2009); declined
United States National Science Foundation East Asia and Pacific Summer Institute in Australia (6/2007-8/2007)
1st place poster: 2006 UCHSC Department of Medicine Research Day (10/2006)
C. Werner and Kitty Hirs 2006 Research Award for UCHSC PhD Student Travel to National Meetings (8/2006)
2nd place poster: 2006 Cell and Developmental Biology and Reproductive Sciences Annual Retreat (8/2006)
United States DOD Breast Cancer Predoctoral Training Award (3/2006-3/2009); $90,000
1st place poster: 2005 Cell and Developmental Biology and Reproductive Sciences Annual Retreat (9/2005)
Avon Foundation Scholar (2004-2007)
United States DOD Breast Cancer Training Grant (9/2003-8/2004)

RESEARCH CONFERENCES ATTENDED & INVITED SEMINARS

2010 Organizer & Poster Presenter-Postdoc/Faculty Research Day, UNC-Chapel Hill, NC
2010 Poster Presenter-AACR-Metastasis Research Society Meeting, Philadelphia, PA
2010 Seminar-Genetics Colloquium, UNC-Chapel Hill, NC
2009 Organizer-Postdoc/Faculty Research Day, UNC-Chapel Hill, NC
2009 Attendee-100th AACR Annual Meeting, Denver, CO
2007 Seminar-National Institutes of Environmental Health Sciences, Research Triangle Park, NC
2007 Seminar-UNC-Chapel Hill, NC

2007 Seminar-University of Adelaide, Adelaide South Australia.
2007 Seminar-Prince Henry's Medical Institute, Melbourne, Victoria Australia.
2007 Poster Presenter-Keystone Symposia: Host cell response to the cancer cell, Keystone, CO, US
2006 Poster Presenter-Gordon Conference: Lymphatic function and disease, Les Diablerets, Switzerland
2005 Speaker-25th Annual San Antonio Breast Cancer Symposia, San Antonio, TX, US
2003 Attendee-Keystone Symposia-Nuclear Receptors: Steroid Hormones, Keystone, CO, US

PEER REVIEWER

Cancer Epidemiology Biomarkers and Prevention (2)
Cancer Research (4)
Environmental Health Perspectives (1)
Journal of Clinical Chimica Acta (1)
Molecular Cancer Research (1)

PEER REVIEWED PUBLICATIONS

Camp JT, Elloumi F, Roman-Perez E, Rein J, Stewart DA, **Harrell JC**, Perou CM, Troester MA. Interactions with fibroblastas are distinct in Basal-like and luminal breast cancers. Mol Cancer Res. Jan; 9(1): 3-13, 2011.

Spillman MA, Manning NG, Dye WW, Sartorius CA, Post MD, **Harrell JC**, Jacobsen BM, Horwitz KB. Tissue- specific pathways for estrogen regulation of ovarian cancer growth and metastasis. Cancer Res. Nov 1, 2010; 89(27);

Pinto MP, Badtke MM, Dudevoir ML, **Harrell JC**, Jacobsen BM, Horwitz KB. Vascular endothelial growth factor secreted by activated stroma enhances angiogenesis and hormone-independent growth of estrogen receptor-positive breast cancer. Cancer Res. April 1, 2010;70(7);2655-64.

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Massart F, Federico G, **Harrell JC**, Saggese G. Growth Outcome during GnRH agonist treatments for slowly progressive central precocious puberty. Neuroendocrinology. 2009;90(3):307-14.

Horwitz KB, Dye WW, **Harrell JC**, Kabos P, Sartorius CA. Rare steroid receptor negative basal-like tumorigenic cells in luminal subtype human breast cancer xenografts. PNAS. 2008 Apr 15;105(15):5774-9.

Harrell JC, Dye WW, Harvell DM, Sartorius CA, Horwitz KB. Contaminating cells alter gene signatures in whole organ versus laser capture microdissected tumors: a comparison of experimental breast cancers and their lymph node metastases. Clin Exp Metastasis. 2008;25(1):81-8.

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SUPPORTING DATA

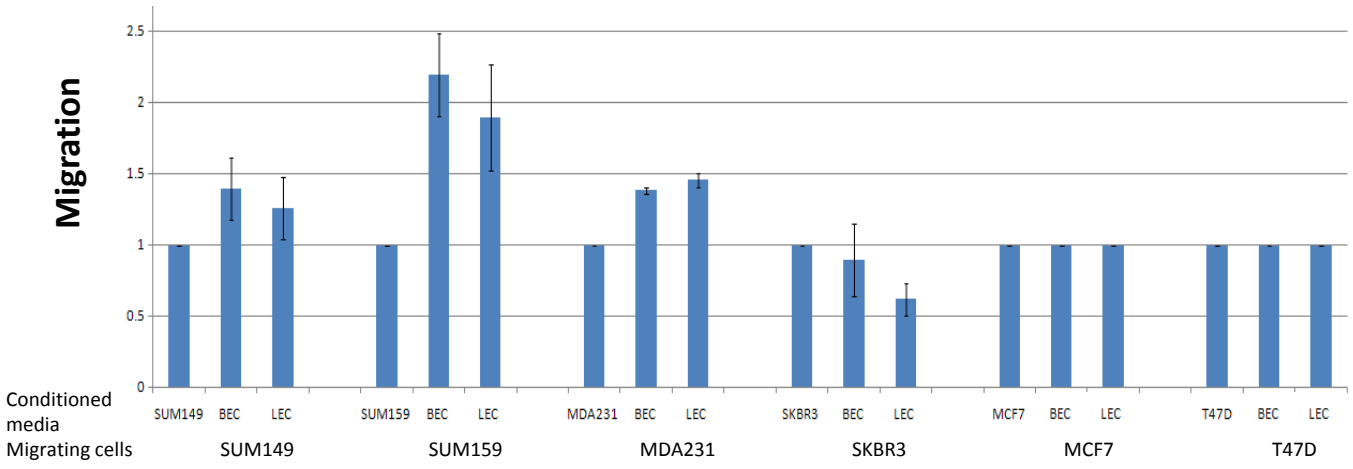
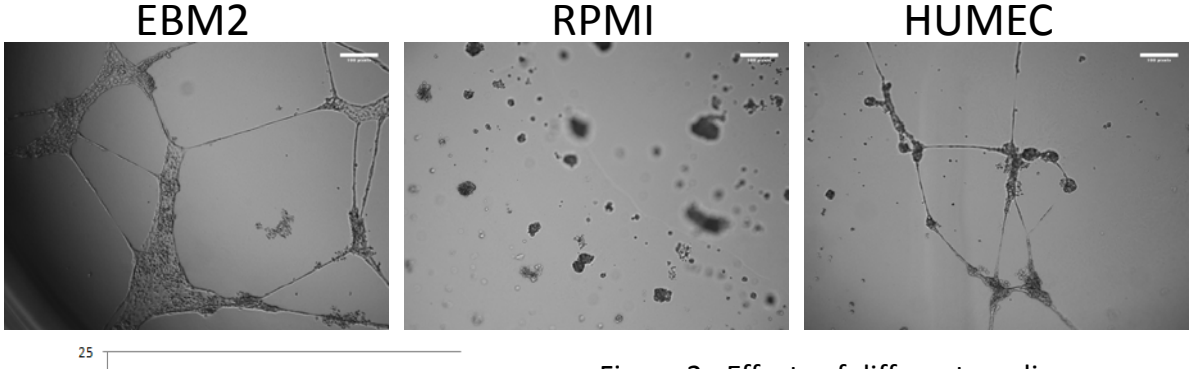


Figure 1: Migratory effects of breast cancer cell—endothelial cell line interactions. Migration assays were performed with endothelial cells and cancer cells in boyden chambers using 100% CM as chemoattractants for 24 hours. Cells were fixed to the boyden chamber membrane, stained with crystal violet, solubilized in methanol and read at 570nm. Absorbance readings were normalized to a value of 1 for each respective cell line.



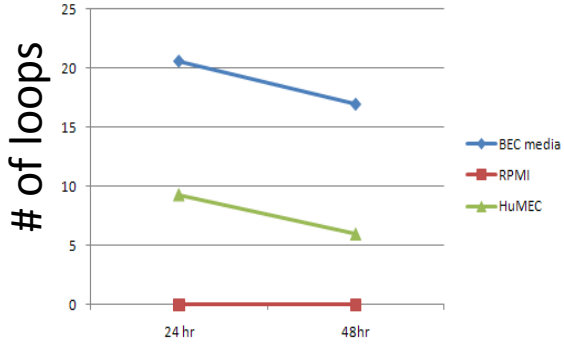


Figure 2: Effects of different medias on endothelial cell 3D tube formation. 25K cells were plated on 100% matrigel and the number of closed loops were counted 24 or 48 hours later. Shown are representative images of multiple experiments.

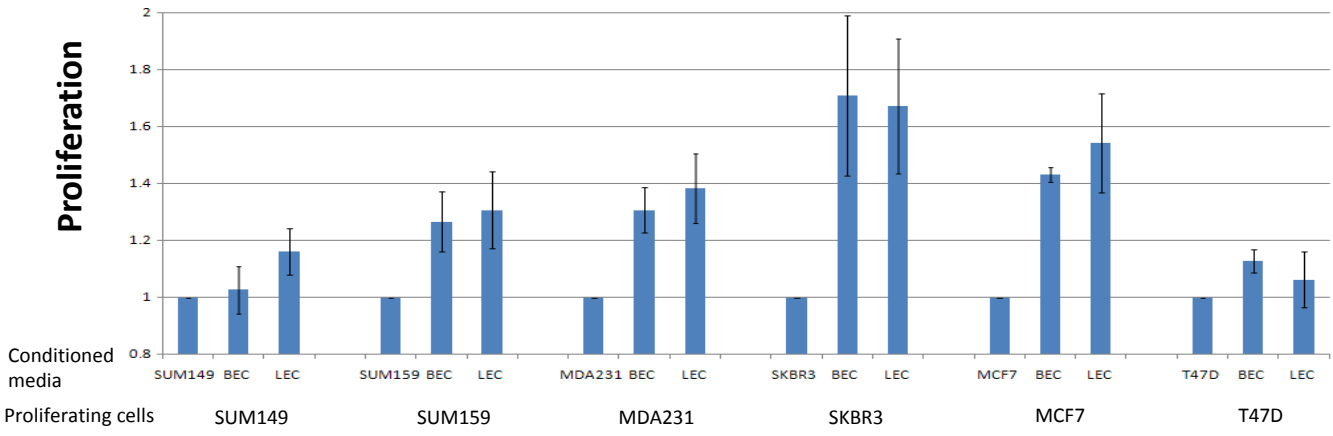


Figure 3: Proliferative effects of breast cancer cell—endothelial cell line interactions. Cancer cell lines representing different intrinsic subtypes were plated in 50% conditioned media (CM) and relative proliferation rates were measured 48 hours later. Each cell line was normalized to its own CM.

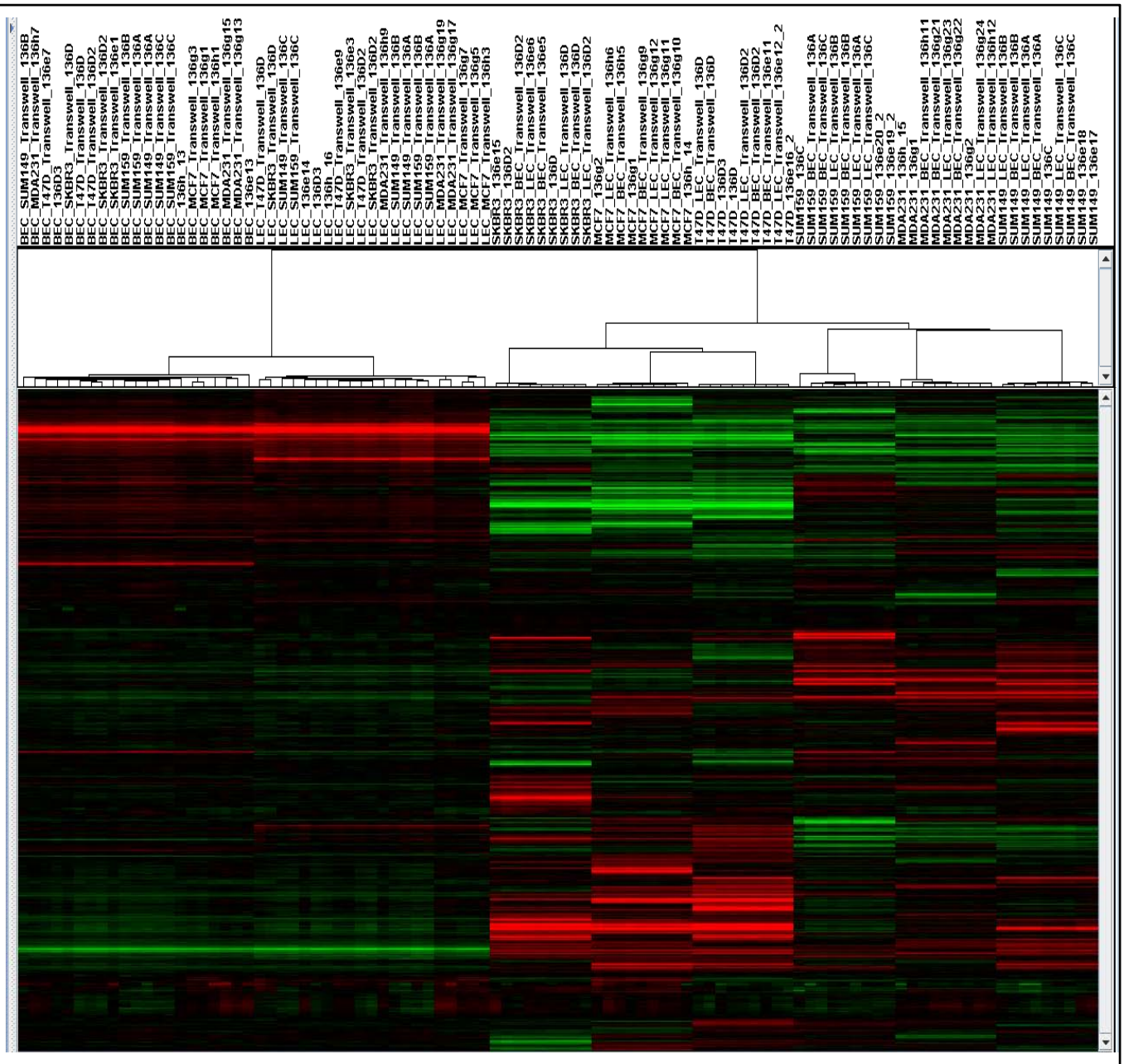


Figure 4: Effects of transwell co-culture on cancer cell and endothelial cell gene expression profiles. Show are all 33,063 genes.

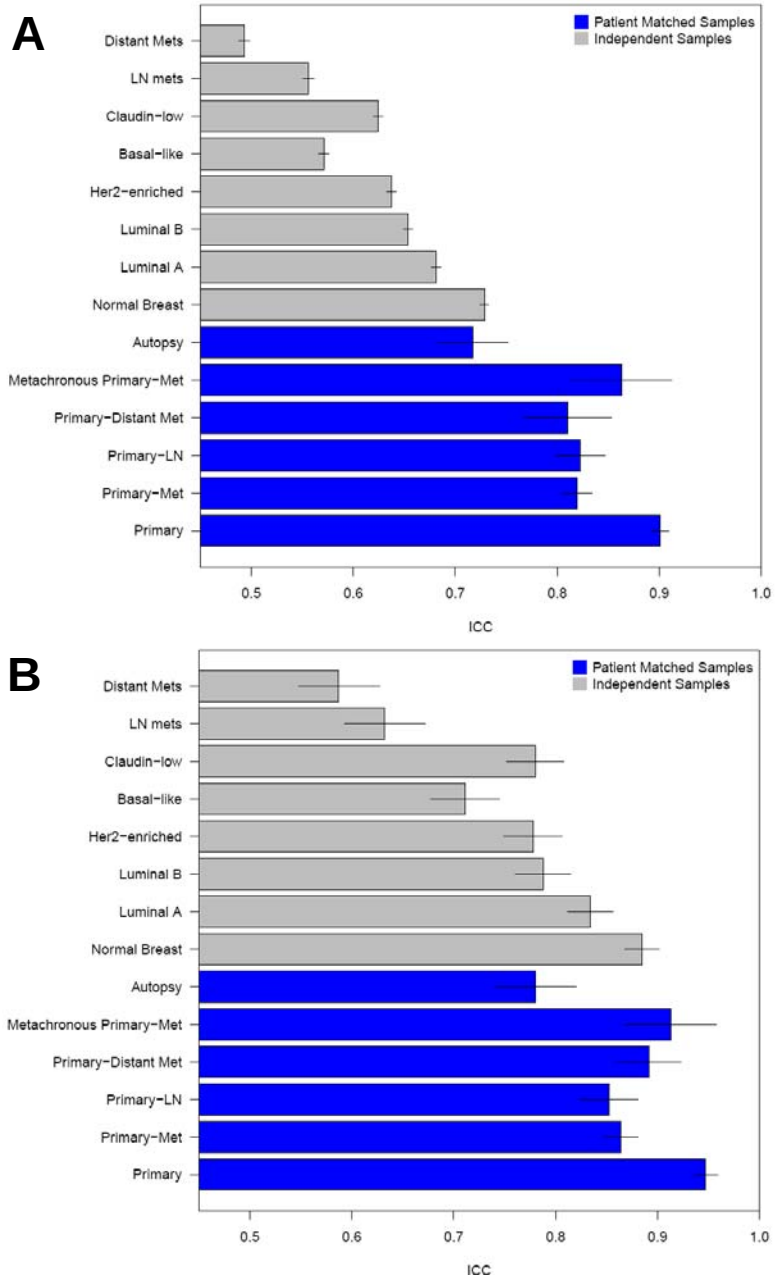


Figure 5: Genomic similarity of breast tumors and metastases. Microarrays were performed on 265 primary tumors and 85 metastases and the overall similarity was measured by intra-class correlation (ICC), with estimates plotted showing 95% confidence intervals. *A*) Using all variably expressed genes, gene expression concordance values were measured in matched samples from the same patient; primary tumors split in 2 (n=40), tumor-metastasis pairs (n=34), tumor-LN metastasis pairs (n=24), tumor-distant metastasis (n=10), autopsy patient metastases from multiple organs within the same patient (n=33), metachronous tumor-metastasis pairs (n=10), or from independent patient samples; normal breast (n=17), Luminal A tumors (n=86), Luminal B tumors (n=50), HER2-enriched tumors n=(25), Basal-like tumors n=(44), Claudin-low tumors (n=45), LN metastases (n=21), and distant metastases (n=45). *B*) ICC of 298 gene expression signatures/modules[11] using the same samples and pairing used in *A*.

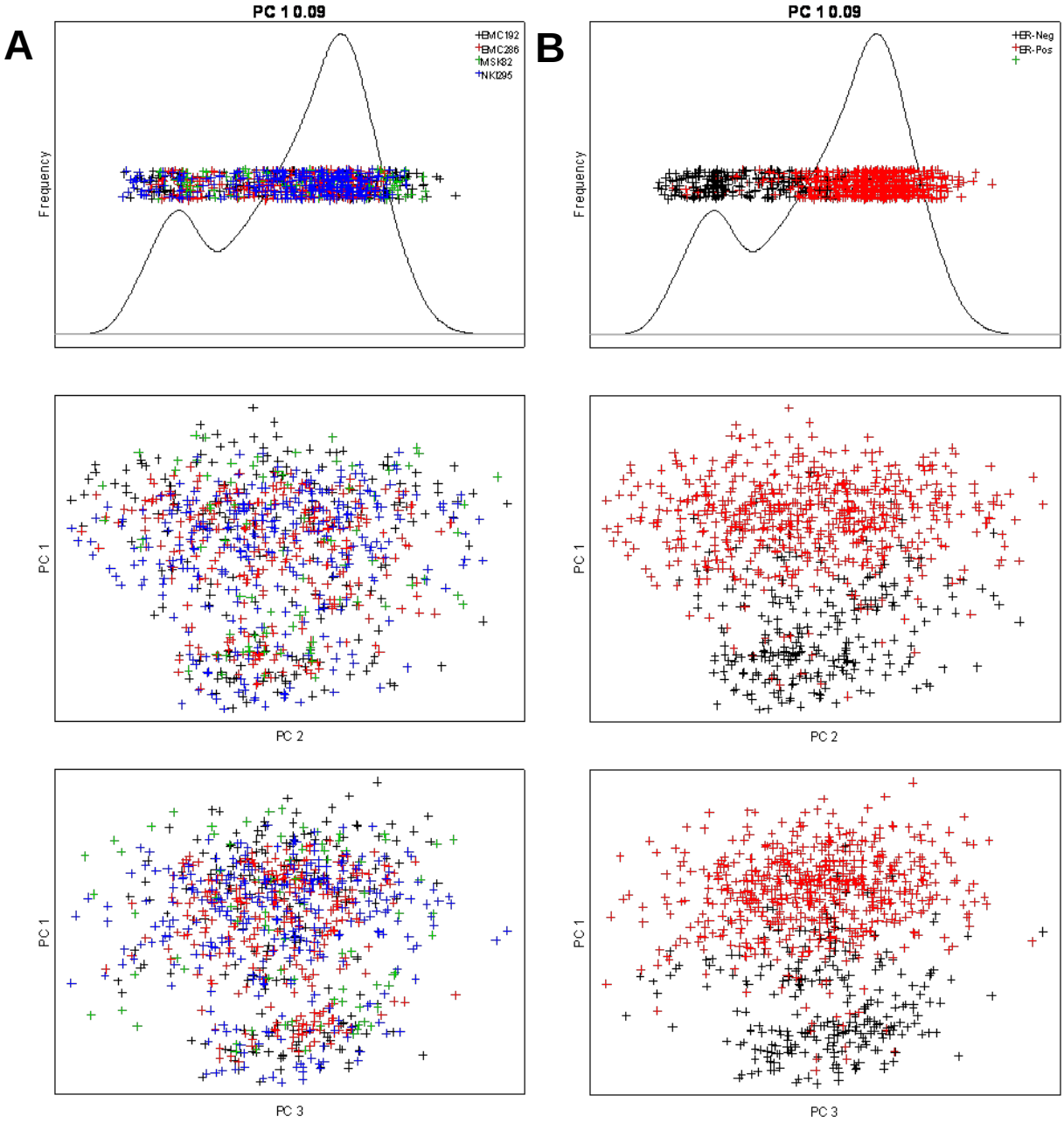


Figure 6: Principal Components Analysis of the 855 combined tumor dataset. After DWD correction, the distribution of samples is shown according to *A*) cohort, or *B*) ER status. The dominant variation is by ER status, not cohort.

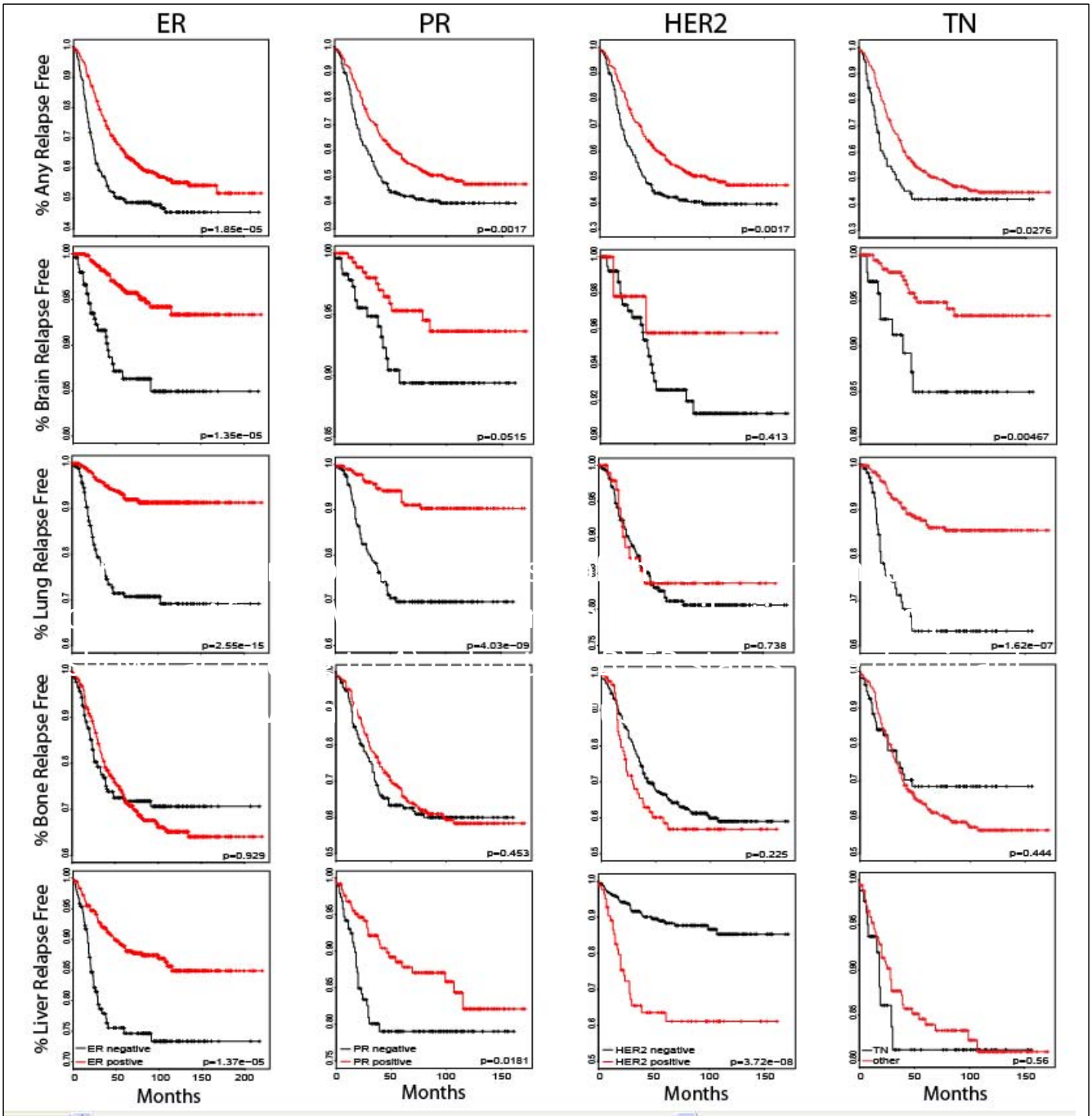


Figure 7: Kaplan-Meier plots for relapse with the 855 tumor dataset according to clinically defined ER status (n=852), PR status (n=537), HER2 (n=499) status, and Triple-Negative status (n=110).

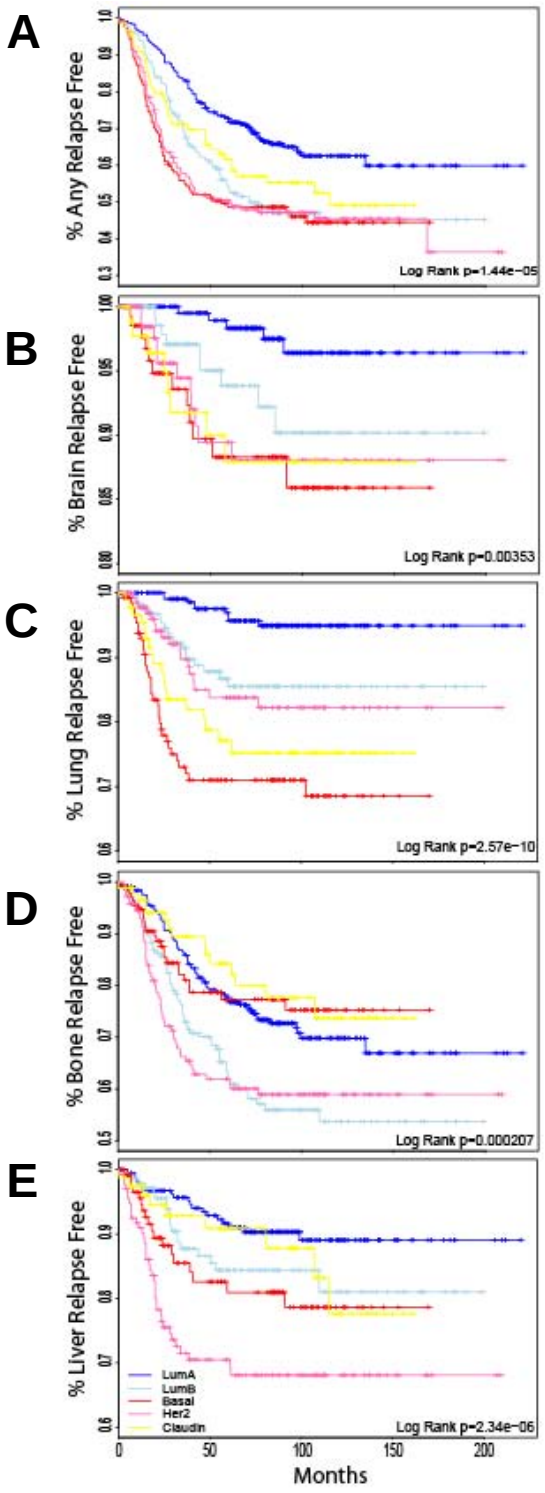


Figure 8: Association of breast cancer subtype with site of first relapse. Shown are Kaplan-Meier plots and Log Rank tests of first site of relapse in each breast tumor subtype in the 779 tumor dataset. If a patient showed 2 or more simultaneous sites of relapse, then this patient was counted as being site of first relapse for both. Organ of first relapse; *A*) Any, *B*) Brain, *C*) Lung, *D*) Bone, *E*) Liver

Cohort	Subtype	# of Tumors	% that Relapsed	Site of first relapse (%)				
				Brain	Lung	Bone	Liver	LN
EMC192	Basal	40	90.0	8.3	41.7	30.6	19.4	NA
	Claudin-low	23	73.9	17.6	41.2	35.3	17.6	NA
	HER2	32	100.0	9.4	18.8	62.5	59.4	NA
	Luminal A	57	89.5	2.0	7.8	76.5	31.4	NA
	Luminal B	31	90.3	3.6	17.9	71.4	14.3	NA
EMC286	Basal	45	37.8	23.5	47.1	41.2	17.6	NA
	Claudin-low	32	28.1	22.2	33.3	44.4	22.2	NA
	HER2	54	38.9	9.5	14.3	76.2	28.6	NA
	Luminal A	72	22.2	0.0	18.8	87.5	0.0	NA
	Luminal B	49	46.9	8.7	34.8	87.0	13.0	NA
MSK82	Basal	17	29.4	20.0	100.0	40.0	NA	NA
	Claudin-low	10	50.0	20.0	100.0	40.0	NA	NA
	HER2	10	20.0	50.0	50.0	50.0	NA	NA
	Luminal A	23	30.4	14.3	14.3	57.1	NA	NA
	Luminal B	16	18.8	0.0	0.0	100.0	NA	NA
NKI295	Basal	38	36.8	28.6	42.9	35.7	57.1	42.9
	Claudin-low	25	28.0	28.6	42.9	42.9	57.1	0.0
	HER2	48	43.8	23.8	33.3	71.4	57.1	28.6
	Luminal A	91	11.0	30.0	10.0	70.0	30.0	30.0
	Luminal B	66	40.9	22.2	18.5	74.1	44.4	25.9
Combined	Basal	140	51.4	16.7	47.2	34.7	28.1	42.9
	Claudin-low	90	42.2	21.1	47.4	39.5	23.1	0.0
	HER2	144	52.8	14.5	22.4	68.4	59.7	28.6
	Luminal A	243	34.6	6.0	10.7	76.2	23.5	30.0
	Luminal B	162	50.0	11.1	22.2	77.8	22.6	25.9
	Any Subtype	779	45.1	12.8	27.4	62.7	29.1	27.8

Table 1: Site of first relapse of the 779 tumors from each cohort according to intrinsic subtype. Several tumors had multiple 1st relapses: Basal-like 22/69, Claudin-low 12/44, HER2-enriched 35/64, Luminal A 19/88, Luminal B 31/86, and these were thus counted as being sites of first relapse for each site.

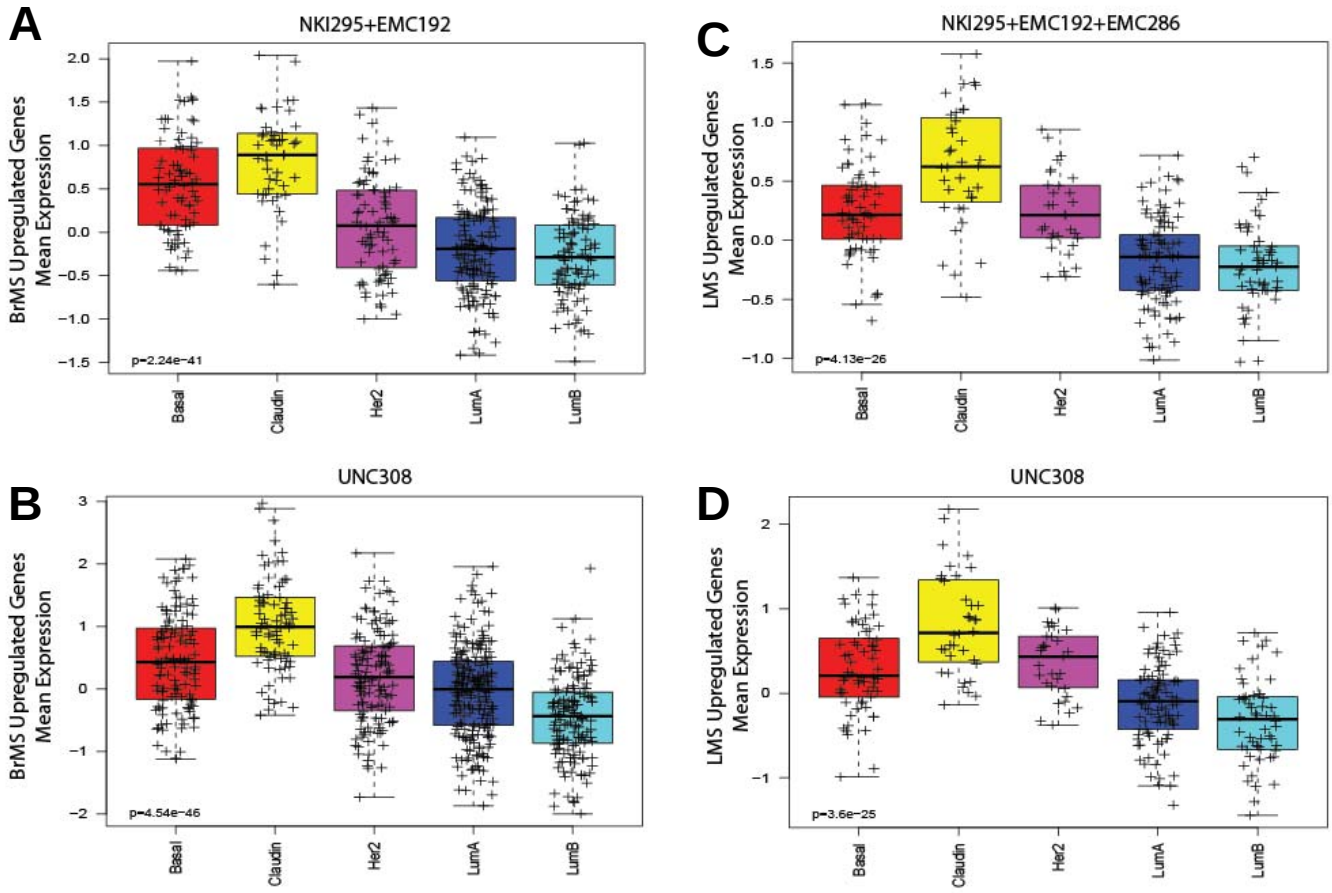


Figure 9: Association of the brain (BrMS) and lung (LMS) cell line based metastasis signatures with intrinsic subtype. Box and whisker plots are shown for each signature on multiple breast tumor microarray data sets according to intrinsic subtype. P-values were calculated with ANOVA. Shown are the same data sets used for the testing of the BrMS (*A*) or LMS (*C*) signatures, as well as an independent UNC dataset (*B, D*).

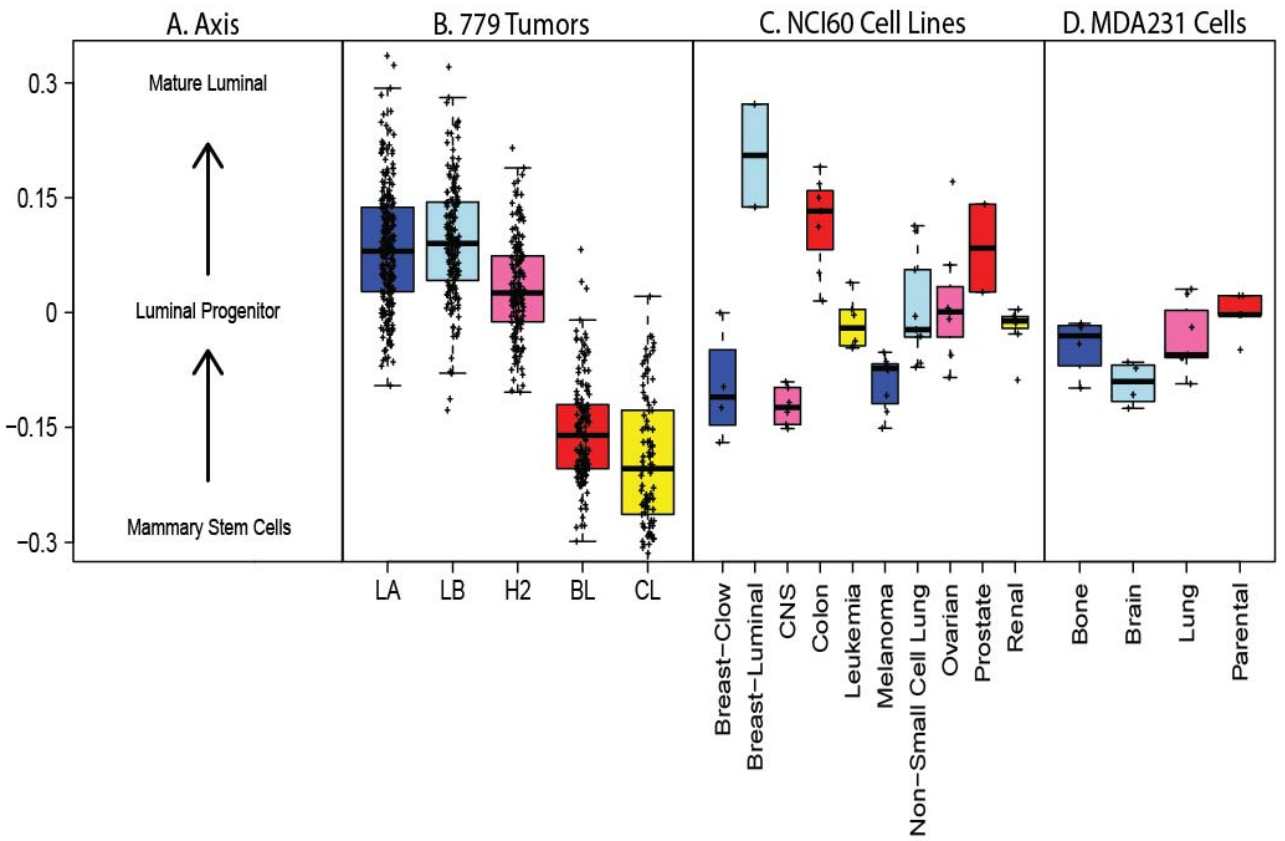


Figure 10: Differentiation Score analysis of the 779 human breast tumors, NCI60 cell lines, and MDA-MB-231 cell lines. *A*) Differentiation axis diagram based on FACS fractions Lim et al., which is described in Prat et al. *B*) Box and whisker plots of the distributions of scores from the 779 tumor dataset according to intrinsic subtype. *C*) NCI60 cancer cell lines gene DS values, with the breast cancer cell lines divided into Claudin-low or Luminal cell lines. *D*) MDA-MB-231 parental, lung-tropic, brain-tropic, and bone-tropic cell lines from the studies of Massague and colleagues.

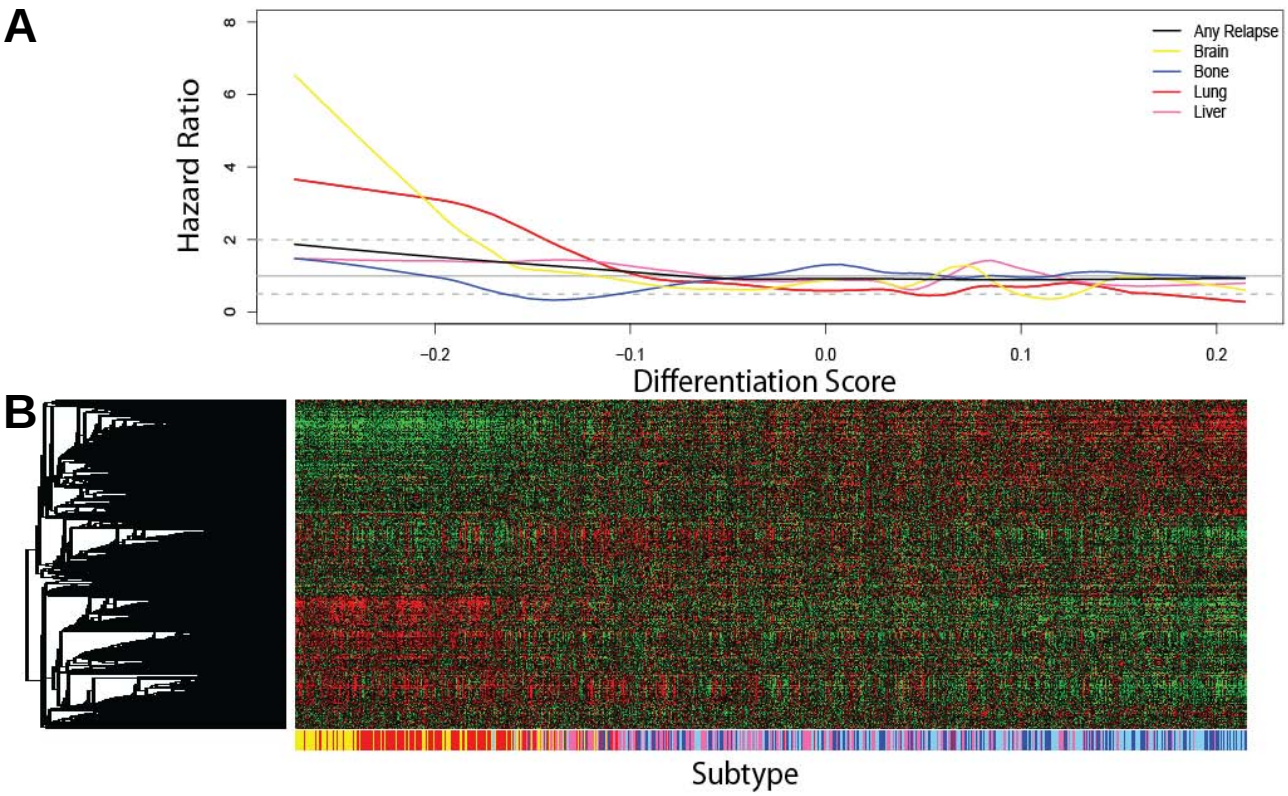


Figure 11: Relationship of Differentiation Score, Breast Cancer Subtype, and likelihood of site of Metastasis. 779 tumors with known first site of relapse were ordered based on low to high DS. *A*) Hazard ratios for each site of metastasis were estimated by grouping a sliding window of 50 samples with consecutive DS and contrasting against those outside the window. Estimates were then smoothed with loess prior to plotting. *B*) Hierarchical clustering of all genes. Below the dendogram is a colored bar identifying the intrinsic subtype of each tumor (yellow; Claudin-low, red; Basal-like; pink, HER2-enriched, dark blue; Luminal A, light blue; Luminal B).

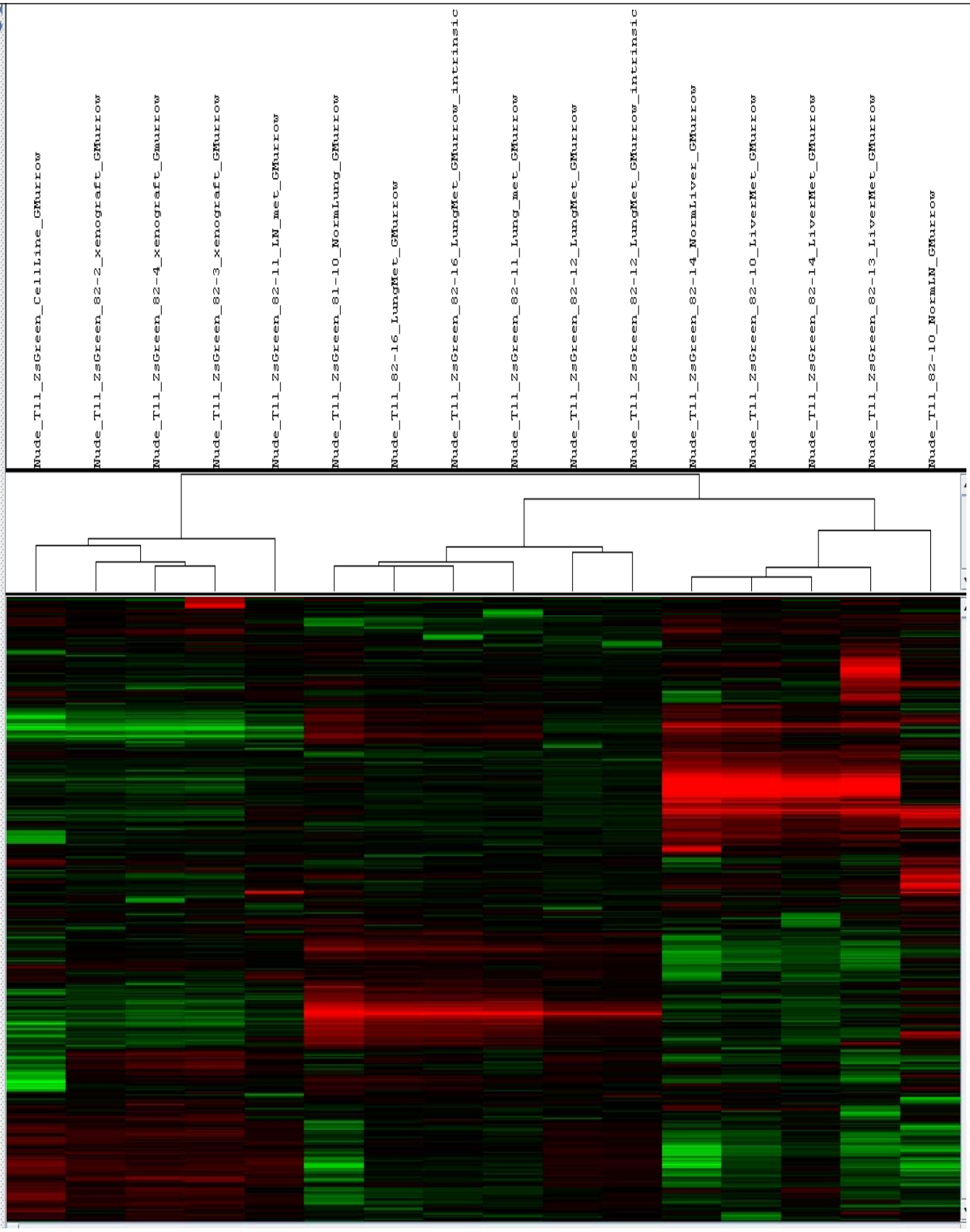


Figure 12: Effect of host tissue cells on cancer gene expression profiles.
Shown are all genes (40,000).